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A STATUS ASSESSMENT OF PERKINSIOSIS, BONAMIOSIS, AND MATEILIOSIS IN COMMERCIAL MARINE BIVALVES FROM SOUTHERN BRAZIL

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ABSTRACT The protozoans *Perkinsus marinus*, *Perkinsus olseni*, *Bonamia ostreae*, *Bonamia exitiosa*, and *Marteilia refringens* are responsible for some of the most detrimental diseases in the production of cultivated shellfish worldwide and are classified as notifiable diseases by the World Organization for Animal Health (OIE). This study examined the general health status of wild and cultured bivalves from southern Brazil and included diagnostic tests for the presence of *Perkinsus* sp., *Bonamia* sp., and *Marteilia* sp. Cultured bivalves included the mangrove oyster *Crassostrea gasar* (syn. *Crassostrea brasiliana*), the brown mussel *Perna perna*, the lion's paw scallop *Nodipecten nodosus*, and wing pearl oyster *Pteria hirundo*. The mangrove oyster *Crassostrea rhizophorae* and the carib pointed venus clam *Anomalocardia brasiliana* (syn. *Anomalocardia flexuosa*) were collected from wild populations. A variety of parasitic or commensal organisms were detected by histology including *Ancistrocoma*-like and *Spenophrya*-like organisms; *Bucephalus* genus, *Nematopsis* sp., *Steinhausia* sp., and *Tylocephalum* sp.; unidentified trematode; unknown protozoan and metazoans; and an amoeba parasite. Hemocytic infiltration was most commonly associated with parasitized animals. Histological, culture and molecular diagnostic tests did not find any evidence of the presence of OIE-listed pathogens or related species in this study. Although the current survey did not identify any pathogens or diseases of concern, it provides baseline health assessment data for these species against which any future disease developments or significant changes in population health can be compared. These data are also valuable with respect to the development and implementation of public policies related to aquatic animal health.

KEY WORDS: OIE diseases, shellfish, pathology

INTRODUCTION

Historically bivalve molluscs such as the zigzag scallop *Euvola* (*Pecten*) *ziczac* (Linnaeus, 1758), carib pointed venus clam *Anomalocardia brasiliana* (Gmelin, 1791) [syn. *Anomalocardia flexuosa* (Linnaeus, 1767)], brown mussel *Perna perna* (Linnaeus, 1758), and the mangrove oysters *Crassostrea rhizophorae* (Guilding, 1828) and *Crassostrea gasar* (Adanson, 1757) [syn. *Crassostrea brasiliana* (Lamark, 1819)] have been important in Brazil as both food and income sources for many coastal communities. Over the past 20 y, the demand for bivalves has increased, which coupled with declining wild harvests has increased interest in bivalve aquaculture, including the introduction and culture of nonnative species such as the Japanese oyster *Crassostrea gigas* (Thunberg, 1793) (Poli 2004).

Bivalve culture in Brazil began in the 1970s, and since 1988 activities have increased to a scale that provides an important alternative source of income to small enterprise fisherman, who often operate as members of associations or cooperatives. Brazilian shellfish culture is dominated by the culture of the brown mussel, followed by the Japanese oyster. There is additional production of native species including the lion's paw scallop *Nodipecten nodosus* (Linnaeus, 1758) and the mangrove oysters *Crassostrea rhizophorae* and *Crassostrea gasar* (FAO 2004). Bivalves are primarily grown in the southern states of Brazil, with Santa Catarina responsible for more than 95% of the total mollusc production (EPAGRI 2014).

Relatively little is known about the health status, pathogens, and parasites of bivalve populations in Brazil (Table 1). The World Organization for Animal Health (OIE)–listed diseases of bivalve molluscs are those which are caused by *Bonamia ostreae*, *Bonamia exitiosa*, *Marteilia refringens*, *Perkinsus marinus*, and *Perkinsus olseni* (OIE 2011). Species of *Perkinsus* have been reported from oysters in the states of Ceará, Paraíba, Bahia, and Sergipe (Sabry et al. 2009, 2013, Brandão et al. 2013b, Da Silva et al. 2013, Queiroga et al. 2013, Da Silva et al. 2014, Da Silva et al. 2015, Queiroga et al. 2015) and in clams from Ceará (Ferreira et al. 2015). Two of these cases represent reports of an OIE-listed *Perkinsus* parasite. Da Silva et al. (2013) reported the presence of *P. marinus* based on rDNA region internal transcribed spacer and large subunit sequences in oysters *Crassostrea rhizophorae*, and Da Silva et al. (2014) and Queiroga et al. (2015) reported *P. marinus* and *P. olseni* by molecular analysis in *Crassostrea gasar*. Both oyster species were collected from Paraiba, northern Brazil. None of the OIE-listed bivalve diseases have been reported from other states in Brazil.

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TABLE 1.

Parasites, pathological conditions, and commensals reported to occur in Brazilian bivalves.

Bivalve species	Parasite, pathological condition or commensal	Author	Site
Anomalocardia brasiliana (syn. *Anomalocardia flexuosa*)	*Bucephalus* sp.	Araújo and Rocha-Barreira (2004), present study	Ceará, Santa Catarina
	Bucephalopsis haimeana	Narchi (1966)	São Paulo
	Cestoda	Boehs and Magalhães (2004)	Santa Catarina
	Encapsulation in gills	Da Silva et al. (2012)	Santa Catarina
	Encysted organisms	Da Silva et al. (2012)	Santa Catarina
	Holothuriophilus tomentosus	Boehs and Magalhães (2004)	Santa Catarina
	Metacercaria	Da Silva et al. (2012)	Santa Catarina
	Nematopsis sp.	Boehs et al. (2010)	Bahia
	Perkinsus beihaiensis	Ferreira et al. (2015)	Ceará
	Polydora (Spionidae)	Boehs and Magalhães (2004), present study	Santa Catarina
	Sphenia antillensis	Boehs and Magalhães (2004)	Santa Catarina
	Steinhausia sp.	Da Silva et al. (2012), present study	Santa Catarina
	Tylocephalum sp.	Boehs et al. (2010), present study	Bahia, Santa Catarina
	Trichodina (genus)	Boehs and Magalhães (2004), Da Silva et al. (2012)	Santa Catarina
	Trematode	Boehs and Magalhães (2004), Boehs et al. (2010), Da Silva et al. (2012)	Bahia
	Turbellarians	Da Silva et al. (2012)	Santa Catarina
	Unidentified metazoans	Boehs et al. (2010)	Bahia
	Unidentified trematode	Da Silva et al. (2012), present study	Santa Catarina
	Unknown ciliates	Boehs and Magalhães (2004), Da Silva et al. (2012)	Santa Catarina
	Unknown metazoan	Present study	Santa Catarina
	Unknown protozoan	Present study	Santa Catarina
Crassostrea gigas	*Ancistrocoma* sp.	Pontinha (2009), Sabry et al. (2011), Da Silva et al. (2012), Ivachuck (2012)	Santa Catarina
	Encapsulation in gills	Da Silva et al. (2012)	Santa Catarina
	Nematopsis sp.	Sabry and Magalhães (2005), Sabry et al. (2011)	Santa Catarina
	Ostracoblabe implexa	Sabry and Magalhães (2005), Pontinha (2009)	Santa Catarina
	Polydora sp.	Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Polydora websteri	Sabry and Magalhães (2005), Pontinha (2009), Da Silva et al. (2012)	Santa Catarina
	Rickettsia-like	Pontinha (2009), Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Steinhausia sp.	Pontinha (2009), Sabry et al. (2011)	Santa Catarina
	Sphenophrya sp.	Pontinha (2009), Sabry et al. (2011), Da Silva et al. (2012), Ivachuck (2012)	Santa Catarina
	Tylocephalum sp.	Sabry and Magalhães (2005), Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Trichodina sp.	Pontinha (2009), Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Turbellarians	Pontinha (2009), Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Unknown ovarian parasite	Da Silva et al. (2012), Ivachuck (2012)	Santa Catarina
	Unknown ciliates	Da Silva et al. (2012)	Santa Catarina
	Unknown metazoans	Pontinha (2009), Da Silva et al. (2012)	Santa Catarina
	Urastoma sp.	Sabry et al. (2011)	Santa Catarina,
	Viral gametocytic hypertrophy	Pontinha (2009), Sabry et al. (2011), Ivachuck (2012)	Santa Catarina

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TABLE 1.

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Bivalve species	Parasite, pathological condition or commensal	Author	Site
Crassostrea rhizophorae	Amoeba (order Dactylopodia)	Sühnel et al. (2014)	Santa Catarina
	Ancistrocoma sp.	Nascimento et al. (1986), Sabry et al. (2011), Zeidan et al. (2012), Sabry et al. (2013), Brandão et al. (2013a), present study	Bahia, Ceará, Santa Catarina
	Bucephalus sp.	Nascimento et al. (1986), Zeidan et al. (2012), Brandão et al. (2013a)	Bahia
	Nematopsis sp.	Nascimento et al. (1986), Sabry and Magalhães (2005), Sabry et al. (2007), Sabry et al. (2011), Da Silva et al. (2012), Sabry et al. (2013), Brandão et al. (2013a), present study	Bahia, Ceará, Santa Catarina, São Paulo
	Ostracoblabe-like	Sabry and Magalhães (2005), present study	Santa Catarina
	Polydora sp.	Sabry et al. (2011), Da Silva et al. (2012), Sabry et al. (2013), present study	Ceará, Santa Catarina
	P. websteri	Sabry and Magalhães (2005)	Santa Catarina
	Perkinsus sp.	Sabry et al. (2009), Sabry et al. (2013), Brandão et al. (2013b)	Bahia, Ceará
	Perkinsus marinus	Da Silva et al. (2013)	Paraíba
	P. beihaiensis	Da Silva et al. (2013), Sabry et al. (2013)	Ceará, Paraíba
	Perkinsus olseni-like	Da Silva et al. (2013)	Paraíba
	Rickettsia-like	Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Steinhausia sp.	Sabry et al. (2011), Sabry et al. (2013)	Ceará, Santa Catarina
	Sphenophrya sp.	Nascimento et al. (1986), Sabry et al. (2011), Zeidan et al. (2012), Brandão et al. (2013a)	Bahia, Santa Catarina
	Tylocephalum sp.	Nascimento et al. (1986), Sabry et al. (2007), Zeidan et al. (2012), Sabry et al. (2013), Brandão et al. (2013a), present study	Ceará, Bahia
	Trichodina sp.	Sabry and Magalhães (2005), Sabry et al. (2011), Sabry et al. (2013), Brandão et al. (2013a)	Bahia, Ceará Santa, Catarina
	Unidentified *Perkinsus* sp.	Da Silva et al. (2012)	Paraíba
	Unknown copepod	Sabry et al. (2011)	Santa Catarina
	Unknown metazoans	Nascimento et al. (1986), Sabry and Magalhães (2005), Da Silva et al. (2012), Brandão et al. (2013a)	Santa Catarina
	Unknown ciliates	Da Silva et al. (2012)	Santa Catarina
	Urastoma sp.	Sabry et al. (2011), Zeidan et al. (2012), Brandão et al. (2013a), Sabry et al. (2013)	Bahia, Ceará, Santa Catarina
	Viral gametocytic hypertrophy	Sabry et al. (2011), Da Silva et al. (2012)	Santa Catarina
	Xenomas	Boehs et al. (2009), Zeidan et al. (2012)	Bahia

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TABLE 1.

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Bivalve species	Parasite, pathological condition or commensal	Author	Site
Crassostrea gasar (syn. *Crassostrea brasiliana*)	Amoeba (order Dactylopodia)	Sühnel et al. (2014), present study	Santa Catarina
	Ancistrocoma sp.	Queiroga et al. (2015), present study	Paraíba, Santa Catarina
	Copepod unknown	Da Silva et al. (2015)	Sergipe
	Maladie du pied shell disease	Da Silva et al. (2015)	Sergipe
	Nematopsis sp.	Queiroga et al. (2015), Da Silva et al. (2015)	Paraíba, Sergipe
	P. marinus	Da Silva et al. (2014), Queiroga et al. (2015)	Paraíba
	P. beihaiensis	Queiroga et al. (2015)	Paraíba
	P. olseni	Da Silva et al. (2014), Queiroga et al. (2015)	Paraíba
	Perkinsus sp.	Queiroga et al. (2013), Da Silva et al. (2015)	Paraíba, Sergipe
	Polydora sp.	Da Silva et al. (2015), present study	Sergipe, Santa Catarina
	Prokaryote-like colonies	Queiroga et al. (2015)	Paraíba
	Steinhausia sp.	Da Silva et al. (2015)	Sergipe
	Sphenophrya-like	Present study	Santa Catarina
	Turbellarians	Da Silva et al. (2015)	Sergipe
	Tylocephalum sp.	Queiroga et al. (2015)	Paraíba
	Viral gametocytic hypertrophy	Queiroga et al. (2015)	Paraíba
	Urastoma sp.	Queiroga et al. (2015)	Paraíba
Iphigenia brasiliana	Unidentified metacercariae	Boehs et al. (2010)	Bahia
	Unidentified metazoan	Boehs et al. (2010)	Bahia
	Tylocephalum sp.	Boehs et al. (2010)	Bahia
Lucina pectinata	No parasite observed	Zeidan et al. (2012)	Bahia
Mytella guyanensis	*Bucephalus* sp.	Ceuta and Boehs (2012), Zeidan et al. (2012)	Bahia
	Nematopsis sp.	Azevedo and Matos (1999), Pinto and Boehs (2008), Boehs et al. (2010), Ceuta and Boehs (2012), Zeidan et al. (2012)	Amazon Estuary, Bahia
	Rickettsia-like	Boehs et al. (2010), Ceuta and Boehs (2012), Zeidan et al. (2012)	Bahia
	Sphenophrya sp.	Zeidan et al. (2012)	Bahia
	Steinhausia mytilovum	Matos et al. (2005)	Amazon Estuary
	Trematode	Boehs et al. (2010),	Bahia
	Unidentified metazoans	Boehs et al. (2010), Zeidan et al. (2012), Ceuta and Boehs (2012)	Bahia
	Urastoma sp.	Zeidan et al. (2012)	Bahia
Nodipecten nodosus	*Polydora* sp.	Present study	Santa Catarina
	Unknown metazoans	Present study	Santa Catarina

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TABLE 1.

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Bivalve species	Parasite, pathological condition or commensal	Author	Site
Perna perna	*Bucephalus* sp.	Umiji et al. (1976), Magalhães (1998), Lima et al. (2001), Da Silva et al. (2002), Galvão et al. (2006), Costa (2007),	Rio de Janeiro, Santa Catarina e São Paulo
	Bucephalus margaritae	Marchiori et al. (2010), Suárez-Morales et al. (2010), Da Silva et al. (2012), Medeiros (2013), present study	Santa Catarina
	Didemnum psammathodes	Suárez-Morales et al. (2010)	Santa Catarina
	Copepoda (*Monstrilla* sp.)	Suárez-Morales et al. (2010)	Santa Catarina
	Nematopsis sp.	Lima et al. (2001), Da Silva et al. (2012), present study	Rio de Janeiro, Santa Catarina
	Polydora sp.	Sabry and Magalhães (2005), Costa (2007), Suárez-Morales et al. (2010), Da Silva et al. (2012), present study	Santa Catarina
	Sphenophrya-like	Present study	Santa Catarina
	Tylocephalum sp.	Da Silva et al. (2012)	Santa Catarina
	Trichodina sp.	Sabry and Magalhães (2005)	Santa Catarina
	Unknown metazoan	Present study	Santa Catarina
	Unknown protozoan in gills	Da Silva et al. (2012)	Santa Catarina
	Unknown ciliates	Da Silva et al. (2012)	Santa Catarina
	Urastoma cyprinae	Suárez-Morales et al. (2010)	Santa Catarina
Pteria hirundo	*Ostracoblabe*-like	Present study	Santa Catarina
	Polydora sp.	Present study	Santa Catarina
	Unknown metazoans	Present study	Santa Catarina
Tagelus plebeius	*Parvatrema* sp.	Da Silva et al. (2009)	Santa Catarina

This study examined wild and cultured populations of economically important bivalves from the state of Santa Catarina, southern Brazil, for general health using histology techniques and for the presence of pathogens responsible for OIE-listed bivalve diseases using culture and molecular methods. Improved understanding of the baseline health status of Brazilian bivalve populations is important and is essential to ensure the continued successful development of the industry and to support the development of appropriate regulatory policies.

MATERIALS AND METHODS

Collection Sites and Processing

A total of 870 bivalves were collected in winter 2009 and summer 2010 from four locations in Santa Catarina, southern Brazil. Wild oysters, *Crassostrea rhizophorae*, were randomly selected from one rocky shore site (approximately 100 m^2) at Praia da Ponta do Sambaqui, Florianópolis (27° 29′ 26.49″ S and 48° 32′ 17.88″ W). This site is approximately 500 m from areas where molluscs are cultured. Carib pointed venus clams *Anomalocardia brasiliana* were collected randomly from one site (approximately 200 m^2) within the Reserva Marinha Extrativista do Pirajubaé (Marine Extractive Reserve of Pirajubaé), Florianópolis (27° 29′ 18.8″ S and 48° 32′ 12.9″ W). This reserve, managed by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, is one of the largest clam harvesting areas in Santa Catarina.

Samples of brown mussels *Perna perna*, mangrove oysters *Crassostrea gasar*, and winged pearl oysters *Pteria hirundo* (Linnaeus, 1758) were obtained from culture systems within the experimental area of the Federal University of Santa Catarina, Ponta do Sambaqui beach (27° 29′ 18.8″ S and 48° 32′ 12.9″ W), Florianópolis. This is the most important area of oyster seed production in southern Brazil. Samples of lantern net–cultured scallops *Nodipecten nodosus* were collected from the experimental area of Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (27° 12′ 5.39″ S and 48° 30′ 47.7″ W). This northern location is an important shellfish production area, especially for *P. perna*.

Live animals were transported on ice to the Nucleus for Aquatic Pathology Study laboratory in the Federal University of Santa Catarina for necropsy and tissue sampling. At necropsy, shell lengths were obtained, and each animal was examined for gross tissue lesions or other signs of disease. Tissue samples of the bivalves for diagnostic testing were removed using sterile techniques and treated as described below.

The number and size of each species examined is provided in Table 2. In the case of wild-collected *Crassostrea rhizophorae* and *Anomalocardia brasiliana*, subsamples of 40 of the 150 individuals examined for gross signs of disease were randomly selected for analysis by hemolymph smears, tissue imprints, and histology (Table 2). A random subset of animals previously sampled for diagnostic testing was also sampled for molecular analysis.

Microscopic Analysis

Ray's Fluid Thioglycollate Culture Method

Rectum and gill tissues were dissected into Falcon tubes containing Ray's fluid thioglycollate culture media (Ray 1954, 1966), to which antibiotics (500 U/ml penicillin, 500 U/ml streptomycin) and an antifungal (nystatin: 10,000 U/ml) were added. Tubes were incubated at room temperature (24.0°C ± 1.5°C) for 5 days in the dark. After incubation, the tissues were removed, macerated onto glass slides, and stained with Lugol's solution. Slides were examined first at 5× objective, then at 100× objective for the detection of hypnospores of *Perkinsus* spp. as recommended by the OIE (OIE 2011).

Tissue Imprints

Small pieces (2 × 2 mm) of heart, gonad, gill, and digestive gland were dried on absorbent paper, and four imprints of each tissue were made onto glass slides. Slides were air-dried, fixed in methanol (1 min), stained with Giemsa (2 min), washed in distilled water (1 min), air-dried, and then mounted with a coverslip using Evermount. Slides were examined first at 40× objective and then under oil immersion at 100× objective for the detection of *Bonamia* spp. and *Marteilia* spp. (OIE 2011).

Hemolymph Smears

Hemolymph samples were obtained from the adductor muscle using 21-gauge (for scallops, mussels, pearl oysters, and true oysters) and 26-gauge needles (for clams) attached to 1-mL sterile syringes. Samples were smeared onto glass slides to form a monolayer, air-dried, and stained with Giemsa as described in section Tissue Imprints. Slides were examined first at 10×, then at 40× objective, and finally under oil immersion at 100× objective for the presence of *Bonamia* spp. and *Perkinsus* spp. (OIE 2011).

Histopathology

To obtain a representative sampling of the visceral mass (which includes gills, gonad, digestive gland, foot, and mantle), a single transverse cross-section (approximately 5 mm thick) was excised from each individual following the procedures outlined in the works of Howard and Smith (1983) and Howard et al. (2004). Tissues were placed in cassettes, fixed for 48 h in Davidson's fixative (Shaw & Battle 1957), dehydrated, and embedded in paraffin. Then 5-μm sections were mounted on slides, deparaffinized, and stained with hematoxylin and eosin. Slides were examined using light microscopy at 10–100× objective for the presence of *Bonamia* spp., *Perkinsus* spp., and *Marteilia* spp. (OIE 2011), as well as signs of disease and/or other pathogens and parasites. The percent prevalence of each parasite was calculated as the number of parasitized animals divided by the total number of each bivalve species multiplied by 100 for each sampling (Bush et al. 1997).

Molecular Analysis: Polymerase Chain Reaction

For the detection of *Bonamia* spp. and *Marteilia* spp. by polymerase chain reaction (PCR), a small piece of gill and digestive gland tissue was preserved in 95% undenatured ethanol. Molecular diagnostic testing was conducted on 60 individuals each of *Perna perna*, *Crassostrea gasar*, *Nodipecten nodosus*, and *Anomalocaria brasiliana* (Table 2).

For each individual a pool consisting of approximately equal amounts of gill and digestive gland was created, and DNA was extracted using DNeasy Blood & Tissue Kit (QIAGEN) following the manufacturer's instructions. Deoxyribose nucleic

TABLE 2.

Sampling details and observations of bivalve molluscs collected in Santa Catarina State, Brazil, during the winter (August 2009) and summer (January 2010).

Species	Age (years)	Site sampling location	Mean shell length ± SD (mm)	Length (*n*)	Season sampling	Hemolymph smears, tissue imprints, and histopathology (*n*)	Gross signs, RFTM, and tissue assay (*n*)	PCR (*n*)
From culture								
Pteria hirundo	3	Sambaqui Beach,	60.18 ± 1.60	30	Summer/2010	30	30	ns
Perna perna	1	Florianópolis/SC (27° 29′	71.11 ± 4.03	40	Winter/2009	40	40	30
		17.73″ S and 48° 32′ 23.88″ W)	70.53 ± 3.63	40	Summer/2010	40	40	30
Crassostrea gasar	1		52.01 ± 8.35	40	Winter/2009	40	40	30
			52.67 ± 4.52	40	Summer/2010	40	40	30
Nodipecten nodosus	1.5	Enterprise for Agricultural	67.08 ± 5.70	40	Winter/2009	40	40	30
		Research and Extension,	66.42 ± 3.85	40	Summer/2010	40	40	30
		Bombinhas/SC (27° 12′ 5.39″						
		S and 48° 30′ 47.7″ N)						
From wild								
Crassostrea rhizophorae	–	Ponta do Sambaqui Beach,	60.55 ± 7.90	150	Winter/2009	40	150	ns
		Florianópolis/SC (27° 29′	61.34 ± 4.54	150	Summer/2010	40	150	
		26.49″ S and 48° 32′ 17.88″ W)						
Anomalocardia brasiliana	–	Marine Extractive Reserve of	21.63 ± 4.29	150	Winter/2009	40	150	30
		Pirajubaé, Florianopolis/SC	22.43 ± 5.31	150	Summer/2010	40	150	30
		(27° 38′ 32.63″ S and 48° 32′						
		01.47″ W)						

Species included the cultured bivalves: winged pearl oyster *P. hirundo*, the brown mussel *P. perna*, the mangrove oyster *C. gasar* (syn. *C. brasiliana*), and the lion's paw scallop *N. nodosus*; and bivalves from wild: the mangrove oyster *C. rhizophorae* and the carib pointed venus *A. brasiliana* clam (syn. *Anomalocardia flexuosa*). ns = not sampled.

acid concentration and purity was measured using a NanoVue spectrophotometer.

For the *Marteilia refringens* analysis, the PCR was performed according to the protocol by Le Roux et al. (2001) and OIE (2011), using the primer set M2A 5′-CCG-CAC-ACG-TTC-TTC-ACT-CC-3′ and M3AS 5′-CTC-GCG-AGT-TTC-GAC-AGA-CG-3′. Each PCR reaction consisted of 48 µl of PCR SuperMix (Applied Biosystems) and 2 µl of DNA at a concentration of 100 ng/µl. Amplification was carried out in a Mastercycler Gradient (Eppendorf) under the following conditions: initial denaturation at 94°C for 10 min, followed by 30 amplification cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final elongation step at 72°C for 10 min. The PCR products were run on 1.5% agarose gels at 100 V for 40 min, and the presence or absence of amplified DNA of the expected product size (412 bp) was assessed by comparison with products obtained for positive controls (plasmid ligations of *Marteilia* type M DNA supplied by Isabelle Arzul, Laboratory for Mollusc Diseases and OIE Reference Laboratory for bonamiosis and marteiliosis, IFREMER, France).

For the detection of *Bonamia* spp., the PCR was performed according to the protocol by Cochennec et al. (2000) and OIE (2011), using the primer set BO 5′-CAT-TTA-ATT-GGT-CGG-GCC-GC-3′ and BOAS 5′-CTG-ATC-GTC-TTC-GAT-CCC-CC-3′. The PCR reaction consisted of 48 µl of PCR SuperMix and 2 µl of DNA at a concentration of 100 ng/µl. Amplification was carried as follows: initial denaturation at 94°C for 5 min, followed by 30 amplification cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final elongation at 72°C for 10 min. The PCR products were run on a 1.5% agarose gel at 100 V for 40 min, and the presence or absence of amplified DNA of the expected product size (300 bp) was assessed by comparison with products obtained from the amplification of positive controls for *Bonamia exitiosa* and *Bonamia ostreae* (plasmid ligations of parasite DNA from each species again supplied as above by Isabelle Arzul).

RESULTS

The animals collected from aquaculture sites ranged in age from 1 to 3 years. The precise age of the wild collected bivalves were unknown, but their size and level of gonad development suggested that they were all adults (Table 2). During necropsy, orange lesions on the gonad of the brown mussel, *Perna perna*, were observed in 25% of the animals collected in both winter and summer. These lesions were not observed in the other studied species.

Ostracoblabe-like condition was observed in the summer 2010 in cultured oysters *Crassostrea rhizophorae* and *Pteria hirundo*, in 1 and 5% of samples respectively (Table 3). There was no evidence of these infections impeding valve closure and there was no damage evident in adjacent tissues.

Polychaetes, genus *Polydora* (Spionidae), were present in all of the bivalve species that were examined, occurring at highest prevalence in *Crassostrea rhizophorae* and *Crassostrea gasar* in both winter and summer samples. Polychaetes were also found albeit at lower prevalences in *Perna perna*, *Nodipecten nodosus* and *Anomalocardia brasiliana* in both seasons, and only occurred in *Pteria hirundo* in low numbers in the summer season (Table 3). Some individuals with the highest infestations also had yellow abscesses in the gonadal tissue and the adductor muscle in association with the mud blisters.

No other shell diseases were observed.

Microscopic Observations and PCR

A high prevalence of haemocytic infiltration (HI) was observed in many of the species examined (Table 3). The highest prevalences occurred in *Anomalocardia brasiliana*, *Crassostrea rhizophorae*, *Crassostrea gasar* and *Perna perna* with 28% to 98% of the animals displaying this condition. In *A. brasiliana* haemocytic infiltrations were associated with *Bucephalus* infections, which were commonly found in the digestive gland, gonad and mantle tissues. These were also associated with unidentified trematode infection in the connective tissues and with an unknown protozoan infection in the gill of *A. brasiliana*. In *P. perna* the haemocytic infiltration was again associated with *Bucephalus* infections, and was observed in 20% of the heavily infected animals (i.e., those with the parasite in more than three different organs) in the winter and 70% of the heavy infected animals in the summer.

During the analysis two ovarian parasites were observed – an amoeba parasite (Figure 1A) and a microsporidian (Figure 1B). The amoeba was found in the oocyte and gonad connective tissues of both oyster species, *Crassostrea rhizophorae* and *Crassostrea gasar*. Full descriptions of this parasite can be found in Sühnel et al. (2014). Da Silva et al. (2012) and Ivachuck (2013) observed a similar parasite described as unknown ovarian parasite in the oyster *Crassostrea gigas*, from the same region (Sambaquí Beach, Florianópolis). The highest prevalence of this amoeba was observed in winter samples of *C. gasar* (Table 3). This amoeba parasite may affect the oocyte viability, impacting reproduction performance, although more analysis would be necessary to fully elucidate the impact of this parasite on the reproductive viability of the host. The other ovarian parasite was a *Steinhausia*-like microsporidian (Figure 1B), which was observed at low prevalence in summer *Anomalocardia brasiliana* clam samples, where it infected the oocyte cytoplasm (Table 3). No host reaction was observed in the infected animals. Sporocysts (diameter: 14.91 ± 0.08 µm) contained spores at different development stages.

The bucephalid infecting the mussel *Perna perna* was identified as *Bucephalus margaritae* (Figure 1C). The species from *Anomalocardia brasiliana* could only be identified to genus as *Bucephalus* sp. (Figure 1D). In both cases the parasite was observed as sporocyst and cercariae stages, according to the description in Marchiori et al. (2010). In both summer and winter samples prevalence was low in *P. perna* mussel samples but higher in *A. brasiliana* clams. 50% of individuals in winter *P. perna* samples had heavy infections (parasite found in more than three organs; i.e. gill, gonad and digestive gland). All summer samples had heavy infections. This was not the case for *A. brasiliana* where 100% of the infected animals showed low-intensity infections in the connective tissue in the winter and summer. Whilst 100% of infections were associated with characteristic orange lesions in *P. perna*, such lesions were not observed in *A. brasiliana* necropsies.

An unidentified trematode (Figure 1E) was observed in the connective tissue of the clam *Anomalocardia brasiliana*.

Nematopsis sp. (Apicomplexa: Eugregarinida) (Figure 1F) were present in 80% and 75% of *Crassostrea rhizophorae*,

TABLE 3.

Percent prevalence of pathology and parasites in bivalves collected from Santa Catarina State, southern Brazil, during the winter 2009 and summer 2010.

Pathological condition and organisms	Winter/2009					Summer/2010					
	Cultured bivalves			Wild-collected bivalves		Cultured bivalves				Wild-collected bivalves	
	P.p.	*C.g.*	*N.n*	*C.r.*	*A.b.*	*P.h.*	*P.p.*	*C.g.*	*N.n*	*C.r.*	*A.b.*
HI	42.5	2.5	2.5	27.5	97.5	5.0	67.5	42.5	2.5	32.5	57.5
Amoeba	0	45.0	0	0	0	0	0	2.5	0	10.0	0
Ancistrocoma-like	0	2.5	0	0	0	0	0	0	0	2.5	0
Bucephalus sp.	0	0	0	0	97.5	0	0	0	0	0	57.5
Bucephalus margaritae	25.0	0	0	0	0	0	25.0	0	0	0	0
Nematopsis sp.	0	0	0	80.0	0	0	0	0	0	75.0	0
Ostracoblabe-like	0	0	0	0	0	5.0	0	0	0	1.0	0
Polydora sp.	12.5	75.0	5.0	84.0	0.3	33.3	22.5	97.5	10.5	96.6	0.5
Sphenophrya-like	2.5*	5.0*	0	0	0	0	0	22.5*	0	0	0
Steinhausia sp.	0	0	0	0	0	0	0	0	0	0	2.5
Tylocephalum sp.	0	0	0	15.0	2.5	0	0	0	0	0	0
Unidentified trematode	0	0	0	0	2.5	0	0	0	0	0	2.5
Unknown metazoan	2.5*	0	0	0	10.0*	0	0	0	0	0	2.5*
Unknown metazoans	5.0	0	7.5*	0	0	7.5*	7.5	0	15.0*	0	0
Unknown protozoan	0	0	0	0	2.5*	0	0	0	0	0	0

Cultured bivalves included brown mussels *Perna perna* (*P.p.*), mangrove oysters *Crassostrea gasar* (*C.g.*) (syn. *brasiliana*), lion's paw scallops *Nodipecten nodosus* (*N.n.*), and wing pearl oysters *Pteria hirundo* (*P.h.*). Wild-collected bivalves were mangrove oysters *Crassostrea rhizophorae* (*C.r.*) and carib pointed venus *Anomalocardia brasiliana* clams (*A.b.*) (syn. *A. flexuosa*).

* Parasite or commensal first time reported for the species.

collected in the winter and summer samples respectively (Table 3), and was most commonly found in digestive glands, with occurrence to a lesser extent in gonad, gill and mantle tissues. No other bivalve species examined in the present study was infected by *Nematopsis* sp.

Ciliates (genus *Sphenophrya*, Figure 1G) were found at low infection rates in gills of summer *Perna perna* samples, and in winter and summer samples of *Crassostrea gasar*. Another ciliate species, genus *Ancistrocoma* (Figure 1H), was found at low prevalence in the digestive gland of *Crassostrea rhizophorae* (summer) and *C. gasar* (winter, Table 3).

Platyhelminth infections (*Tylocephalum* sp.; Figure 1I; mean ± standard deviation sizes 97.66 ± 0.04 μm by 80.34 ± 0.08 μm) were observed in the connective tissues of *Anomalocardia brasiliana* and *Crassostrea rhizophorae* winter samples, with a higher prevalence seen in the latter (Table 3).

An unknown metazoan (Figure 1J) was observed in the gill filaments of *Anomalocardia brasiliana* in an encapsulated form, with higher winter prevalence than in summer samples. This parasite was also observed in low levels in the gill filaments of *Perna perna* winter samples (Table 3), but at a different development stage, with the diameter ranging from 135.36 to 190.15 μm.

Three other unidentified metazoans were observed at low prevalence in different species in this study. In *Pteria hirundo* an unknown metazoan (99.47 ± 2.49 μm diameter, Figure 1L) was found encapsulated in the digestive gland. In *Perna perna*, an unknown metazoan (93.00 ± 8.11 μm diameter; Figure 1M) was observed encapsulated in the connective tissue of the mussel. An unknown metazoan (Figure 1N) in the lumen of the intestine and stomach of *Nodipecten nodosus* was found.

RFTM assays (*Perkinsus* spp.), tissue imprints (*Bonamia* spp. and *Marteilia* spp.), haemolymph smears (*Bonamia* spp. and *Perkinsus* spp.) and PCR analysis (*Bonamia exitiosa*, *Bonamia ostreae* and *Marteilia refringens*) showed all negative results for the studied bivalves.

DISCUSSION

Ostracoblabe-like condition observed in the present study showed similar characteristics described by Bower (2001), where nacre covered bumps which protrude from the shell into the adductor muscle are a typical sign of infection with the fungus *Ostracoblabe implexa*. Other shell diseases observed in the present study, *Polydora*, was observed by Sabry and Magalhães (2005) causing mud blisters in the bivalve shells, with 100% of the infected animals, and are known to be associated with polychaete infections.

A high prevalence of haemocytic infiltration (HI) was observed in many of the species examined (Table 3). The highest prevalences occurred in *Anomalocardia brasiliana, Crassostrea rhizophorae*, *Crassostrea gasar* and *Perna perna* with 28% to 98% of the animals displaying this condition. The high haemocytic infiltration observed in *C. rhizophorae* and *C. gasar* could be related to gonad parasites infection as observed by Sühnel et al. (2014). In *P. perna*, infiltration was linked to unknown metazoans, but was also observed in unparasitized mussels, and may be related to reproductive physiology or to an unidentified potential agent.

Ovarian parasites observed in the present study as similar those observed by Da Silva et al. (2012) and Ivachuck (2012), described as unknown ovarian parasite in the oyster *Crassostrea*

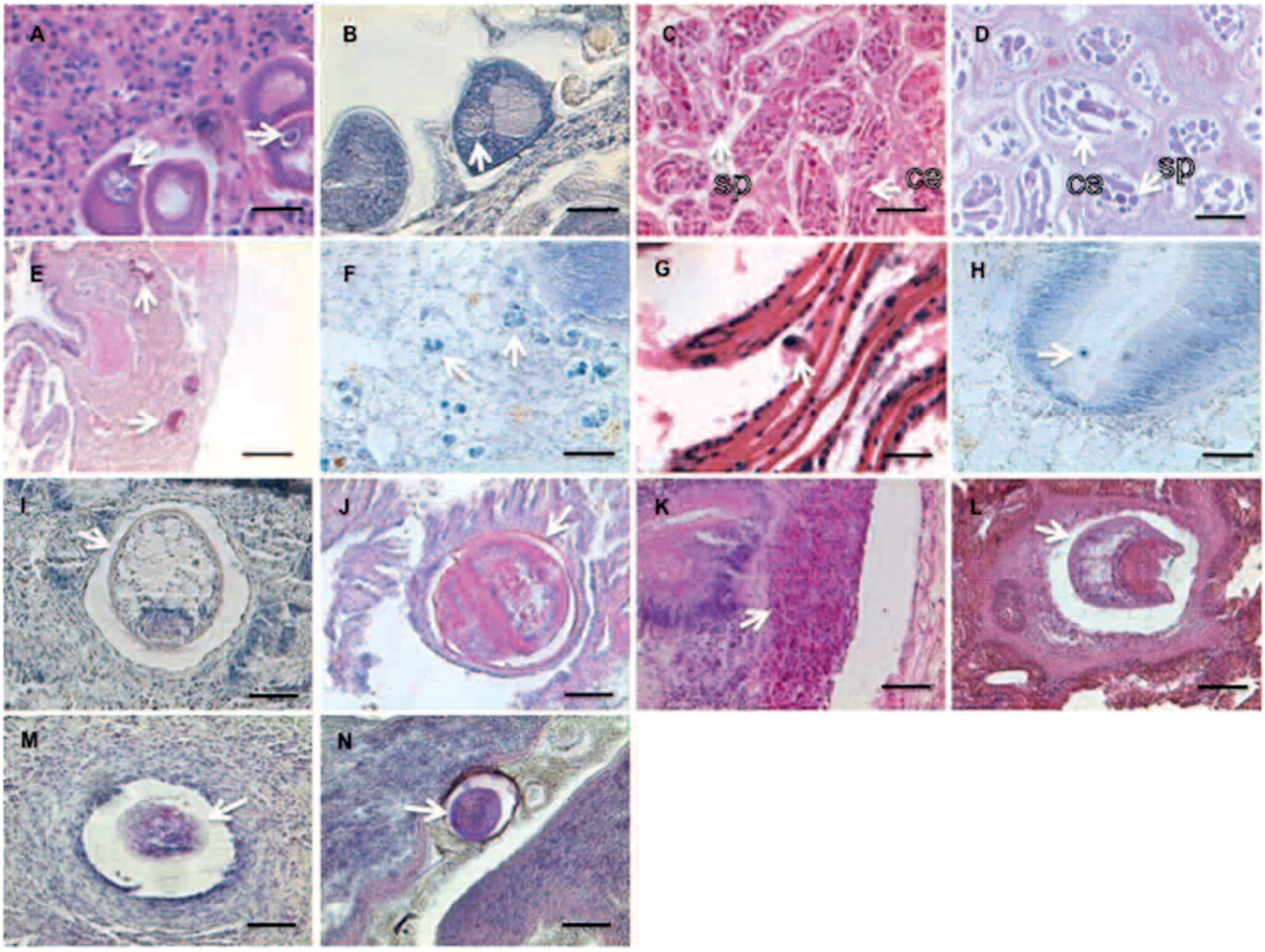

Figure 1. Histological section (hematoxylin and eosin stain) of bivalves molluscs from Santa Catarina, Brazil: (A) unknown ovarian parasite in the oocyte cytoplasm of the oyster *Crassostrea gasar*, (B) *Steinhausia* sp. sporocyst in the oocyte cytoplasm of the clam *Anomalocardia brasiliana*, (C) *Bucephalus margaritae* infecting the gonad tissue of the mussel *Perna perna*, (D) *Bucephalus* sp. infecting the gonad tissue of the clam *A. brasiliana*, (E) unidentified trematode infecting the connective tissue of the clam *A. brasiliana*, (F) *Nematopsis* sp. oocysts in the connective tissue of the oyster *Crassostrea rhizophorae*, (G) *Sphenophrya*-like ciliate in the gill of the oyster *C. gasar*, (H) ciliate *Ancistrocoma* sp. in the digestive gland of the oyster *C. rhizophorae*, (I) *Tylocephalum* sp. in the connective tissue of the clam *A. brasiliana*, (J) unknown metazoan infecting the gill filament of the clam *A. brasiliana*, (K) unknown protozoan infecting the connective tissue of the clam *A. brasiliana*, (L) unknown metazoan infecting the digestive gland of the pearl oyster *Pteria hirundo*, (M) unknown metazoan infecting connective tissue of the mussel *P. perna*, and (N) unknown metazoan infecting the digestive gland of the scallop *Nodipecten nodosus*. Scale bar in A and B: 200 μm (100× objective); scale bar in C, D, E, H, I, J, K, L, M, and N: 50 μm (40× objective); scale bar in F: 50 μm (10× objective); scale bar in G: 20 μm (100× objective); sp: sporocyst; ce: cercariae; arrows: parasites.

gigas, from the same region (Sambaquí Beach, Florianópolis). The other ovarian parasite, *Steinhausia*-like, has been observed in Brazil in the oysters *C. gigas* (Pontinha 2009, Sabry et al. 2011) and *Crassostrea rhizophorea* (Sabry et al. 2011), in the mussel *Mytella guyanensis* (Matos et al. 2005), and in the clam *Anomalocardia brasiliana* (Da Silva et al. 2012).

As bucephalids are often highly specific for their host species (Lauckner 1983), more studies are required to determine which species is associated with *Anomalocardia brasiliana* infections. Buchephalosis has been reported in the *A. brasiliana* clam in Brazil (Narchi 1966, Araújo & Rocha-Barreira 2004, Boehs & Magalhães 2004, Boehs et al. 2010, Da Silva et al. 2012), as well as in the mussel *Perna perna* (Umiji et al. 1976, Magalhães 1998, Lima et al. 2001, Da Silva et al. 2002, Galvão et al. 2006, Costa 2007, Marchiori et al. 2010, Da Silva et al. 2012). In this study bucephalosis was not observed in oysters (*Crassostrea rhizophorae, Crassostrea gasar, Pteria hirundo*) or the scallop (*Nodipecten nodosus*) (Table 3), but it has been reported in *C. rhizophorae* in more northerly sample sites by Nascimento et al. 1986 (São Paulo State). Bucephalid parasites have also been found in two commercially important fishery species, *Mytella guyanensis* (Boehs et al. 2010, Ceuta & Boehs 2012) and *Lucina pectinata* (Oliveira 2008) in Bahia. Bucephalids are known to cause parasitic castration in molluscs (Shelley 1988), which can affect the reproductive capacity of individuals in naturally recruiting wild populations.

The unidentified trematode observed in *Anomalocardia brasiliana* as similar those registered by Da Silva et al. (2012), with encysted phases in the kidney and mantle of *A. brasiliana*.

Histological signs of infection with *Nematopsis* sp., as registered in the present study, include oocysts with an irregular outline that are bound by a single thickened membrane (Padovan et al. 2003). These protozoa use bivalves as intermediate hosts for gametogonic and sporogonic life stages and complete their life cycle in the digestive tract of crustaceans. In the present study, there was little evidence of histopathological changes in any infected tissues, even for animals with more intense haemocytic infiltration. The infection of *Crassostrea rhizophorae* with *Nematopsis* sp. is not unusual as previous reports have found oocysts of this species ranging from 60% to 100% (Sabry et al. 2007) in a Jaguaribe River Estuary population (mainly in mantle and gill tissues), and 14% in subepithelial gill tissues in a northeastern population in Recife (Azevedo et al. 2005).

This is the first time that these *Sphenophrya*-like ciliate have been reported in the *Crassostrea gasar* oyster. While ciliates are commensal organisms, feeding from suspended particles in the gill and mantle of bivalves in this case (Kinne 1983), their presence in high abundance or during periods of environmental and/or physiological stress can cause damage to the host organism (Lauckner 1983).

An unknown metazoan (Fig. 1J) was observed in the gill filaments of *Anomalocardia brasiliana* in an encapsulated form, with higher winter prevalance than in summer samples. This parasite was also observed in low levels in the gill filaments of *Perna perna* winter samples (Table 3), but at a different development stage, with the diameter ranging from 135.36 to 190.15 μm.

The unknown metazoan infecting *Anomalocardia brasiliana* observed, is morphologically identified as a copepod, but other authors studying gastropod gill lamellae suggest a similar encapsulated organism may be a metacercarian cyst (De Vico & Carella 2012). This is the first time that this unknown metazoan in *A. brasiliana* has been observed parasitizing the bivalves *A. brasiliana* and *Perna perna*. This study is also the first reported case of this unknown protozoan in the connective tissue and gill filaments of *A. brasiliana*, although a similar unidentified protozoan has been reported in the gill filaments of the mussel *P. perna* by Da Silva et al. (2012). Also the unknown metazoan observed in *Nodipecten nodosus* is the first known occurrence.

In general the parasites and commensal species found in this study have been previously reported in bivalves from Brazil and elsewhere, and are generally considered benign (Bower et al. 1992, Magalhães & Ferreira 2006). The exceptions for this are the ovarian parasites and *Bucephalus* infections, which are known to affect the bivalve reproduction. More research into the unknown parasites observed in this study is needed to identify possible host relationships, using specific techniques such as *in situ* hybridization, molecular analysis and electron microscopy.

About OIE diseases, other studies have examined bivalves collected in the vicinity of the sample sites for the presence of *Perkinsus* species. *Crassostrea rhizophorae* collected from Sambaqui Beach and Ribeirão da Ilha, Florianópolis (SC) were reported to be negative for the parasites *Perkinsus* spp. and a *Perkinsus beihaiensis*-like species using the RFTM assay, whereas oysters collected in the estuary of the River Package, Fortaleza (CE), in northeastern Brazil tested positive (Sabry et al. 2009, Sabry et al. 2013). Also in Bahia, Brandão et al. (2013b) confirmed the presence of *Perkinsus* spp., by RFTM and PCR assays, which had infected the rectum, intestine and stomach of *C. rhizophorae* oysters. Da Silva et al. (2012) did not find *Perkinsus* spp. using RFTM assays in a range of the present studied bivalve species from Santa Catarina (*Perna perna, C. rhizophorae, Crassostrea gigas* and *Anomalocardia brasiliana*). In addition, these authors also examined two specimens of *Crassostrea gigas* for the presence of *Marteilioides chungmuensis*, both of which tested negative for this pathogen. Based on the histological and tissue imprint examinations, no *Marteilioides refringens* or *Marteilia*-related species were present in the bivalves tested. Furthermore the *M. refringens*-specific PCR results were all negative. Although species of *Marteilia* have been reported from a wide variety of bivalves and geographical locations, this genus has never been reported in South American waters (Table 1; Berthe et al. 2004).

The present study found no evidence of *Bonamia* species in the samples following examination of haemolymph samples, tissue imprints and histological sections. PCR results using a primer set specific for *Bonamia ostreae* and *Bonamia exitiosa* were also all negative. The OIE lists all susceptible hosts of *B. exitiosa* and *B. ostreae* as all species within the genus *Ostrea*. Bonamiosis has been reported in *Ostrea chilensis* from New Zealand (Dinamani et al. 1987, Doonan et al. 1994, Hine and Jones 1994, Hine 1996), in *Ostrea angasi* from Australia (Corbeil et al. 2006) *Ostrea stentina* from Tunisia (Hill et al. 2010) and in *Ostrea edulis* from Europe (Abollo et al. 2008, Carrasco et al. 2012). Also, bonamiosis has been reported in the genus *Crassostrea*, where *Crassostrea ariakensis* was infected by *Bonamia exitiosa* (Dungan et al. 2012). Although *Bonamia* have not been reported in Brazilian bivalves, it is important to note that oysters within the genus *Ostrea* have not been examined in Brazil. The puelche oyster, *Ostrea puelchana,* is found in the waters of southern Brazil, has been reported to be a host of *Bonamia* spp. based on collections made in Northern Argentina (Kroeck & Montes 2005, Kroeck 2010). *Bonamia* spp. has also been reported from the cultured Chilean oysters, *Ostrea chilensis*, in their native Chile (Campalans & Lohrmann 2009).

The results of this study support other survey work conducted over the past several years that have examined the health and disease status of bivalves in Santa Catarina State (Lima et al. 2001, Da Silva et al. 2002, Sabry 2003, Boehs & Magalhães 2004, Sabry & Magalhães 2005, Garcia & Magalhães 2008, Pontinha 2009, Sabry et al. 2009, Marchiori et al. 2010, Sabry et al. 2011, Da Silva et al. 2012, Ivachuk 2013). This work contributes to baseline health assessments of *C. gasar* (syn. *C. brasiliana*), providing first reports of *Sphenophrya*-like ciliate infections. It has also provided health data on unknown and unidentified parasites or commensal organisms in a range of bivalve species in Brazil, including new reports as for *Anomalocardia brasiliana*, *Crassostrea gasar* and *Pteria hirundo*.

Based on the wild and cultured populations studied here, no OIE-notifiable bivalve diseases were found in the state of Santa Catarina in southern Brazil in the following species: *Anomalocardia brasiliana* (syn. *A. flexuosa*), *Crassostrea gasar (syn. brasiliana)*, *Nodipecten nodosus* and *Perna perna*. Examination of the wild populations found no OIE-notifiable diseases, suggesting that they may be unlikely to be a harbouring source of disease transmission to cultured populations. Although the current survey did not identify any pathogens or diseases of concern, it provides important baseline health assessment data

for these species against which any future disease developments or significant changes in population health can be compared. The results of the present study support the Ministry of Fisheries and Aquaculture in Brazil (Ministério da Pesca e Aquicultura - MPA) in the development and implementation of public policies related to aquatic animal health. Currently reference laboratories are being developed for the diagnosis of OIE diseases for molluscs, as part of a National Network of Laboratories of the MPA (Rede Nacional de Laborátorios do Ministério da Pesca e Aquicultura - RENAQUA), which was created in April of 2012 in Brasilia.

Continued monitoring along the Brazilian coast (which would include the development of sampling programs covering broader geographical regions, times of the year, and also be extended to include other bivalve species) would lead to a more comprehensive overview of the health of bivalve populations. This information could then be used to inform managers and producers to enable the environmentally responsible aquaculture development of these economically and socially important species.

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